HER2 Assessment by Immunohistochemical Analysis and Fluorescence In Situ Hybridization

Comparison of HercepTest and PathVysion Commercial Assays

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Abstract

We determined HER2 protein overexpression by immunohistochemical analysis and HER2 gene amplification by fluorescence in situ hybridization (FISH) in 215 formalin-fixed, paraffin-embedded breast tumors. Pathologist concordance for immunohistochemical scoring, and HER2 status concordance, as determined by immunohistochemistry and FISH, were high for immunohistochemical 3+, 1+, and 0 tumors but poor for 2+ tumors. Consensus immunohistochemical scores correlated with absolute and chromosome 17 (CEP17)–corrected HER2 gene copy number. Among HER2-nonamplified tumors, the immunohistochemical score and mean absolute chromosome 17 (CEP17) copy number were weakly correlated. Seventeen tumors were HER2-amplified using absolute HER2 gene criteria but nonamplified when corrected for chromosome 17 polysomy (8 of these were immnuohistochemical 2+). Assessment of benign epithelium within the immunohistochemical slides revealed either no staining or basolateral membrane staining, suggesting normal HER2 protein expression. Twenty tumors showing similar basolateral HER2 immunostaining were all low-moderate grade, tubule-forming, and HER2-nonamplifed (17) or borderline amplified (3). Additional studies relating changes in HER2 gene content due to amplification or chromosome 17 polysomy and HER2 protein expression may be helpful to pathologists who interpret HER2 immnuohistochemical slides. Breast tumors scored at 2+ should be analyzed by FISH, preferably using a dual-probe FISH assay capable of distinguishing HER2 gene amplification from chromosome 17 polysomy.

The human HER2 gene (HER-2/*neu*, c-*erb*B-2) encodes 1 member of a family of 4 transmembrane tyrosine kinases (HER1-4), the prototype of which is the epidermal growth factor receptor (HER1, erbB-1) (reviewed in Yarden and Sliwkowski[1]). Various stromal-derived ligands, including epidermal growth factor, epidermal growth factor–like ligands, and neuregulins bind HER1, HER3, and HER4, inducing homodimerization and heterodimerization, phosphorylation of cytoplasmic tyrosine kinase moieties, and activation of complex signaling pathways essential for cell survival, differentiation, and proliferation.[1-4] HER2, however, is an orphan receptor with no known high-affinity ligand. HER2 becomes activated by heterodimerization after direct ligand binding by HER1, HER3, or HER4. Thus, the role of HER2 in the network of membrane receptor kinases seems to be as an amplifying coreceptor for HER1, HER3, and HER4.[2,3] A specific erbB-2 interacting protein (ERBIN) restricts the spatial distribution of the HER2 molecule to the basolateral membrane of epithelial cells.[5] ERBIN binds HER2, but not HER1, HER3, or HER4, and may be involved in connecting HER2 to cytosolic and cytoskeletal-associated components.[5]

The HER2 gene is central to the oncogenesis and clinical behavior of 25% to 30% of human breast cancers.[6,7] HER2 overexpression and/or gene amplification is prognostic for node-positive[6,7] and node-negative breast cancers,[8] predictive for some chemotherapeutic and hormonal agents, and is an indication for trastuzumab (Herceptin, Genentech, South San Francisco, CA) therapy in patients with metastatic breast carcinoma.[9,10] The risk of cardiac toxic effects[11] and the specter of patients with false-positive results accruing into breast cancer clinical trials[12] have recently focused attention

on the sensitivity and specificity of clinical HER2 assays. As the level of HER2 protein overexpression and gene amplification have prognostic and predictive relevance, HER2 assays must be semiquantitative and reproducible and discriminate normal from cancer-associated HER2 protein expression or gene content.[13-15]

For clinical HER2 determination, tissue-based methods, such as immunohistochemical analysis and fluorescence in situ hybridization (FISH), have replaced whole-tissue extraction methods, such as Southern blot analysis, enzyme-linked immunosorbent assay, and polymerase chain reaction, which may require fresh tissue or suffer dilution owing to admixing of tumor and normal cells.[16] Frozen section immunohistochemical analysis, a "gold-standard" method for HER2 overexpression, is impractical in the current era of early cancer detection, in which tumor size often precludes ancillary testing of fresh tissue.

Immunohistochemical analysis is an attractive method for clinical HER2 determination owing to its retrospective potential and specific targeting of tumor cells. The plethora of available antibodies, methods, and grading schemes,[8,17] however, have made standardization impossible. HercepTest (DAKO, Carpinteria, CA), a formalin-fixed, paraffin-embedded (FFPE)-suitable commercial immunohistochemical analysis assay, gained approval from the US Food and Drug Administration (FDA) in 1998 in part for its promise of standardization.[16] Subsequent reports documented low specificity of the HercepTest 2+ category compared with gene amplification assays such as FISH,[14,18,19] some authors even calling for elimination of the 2+ category as a criterion for trastuzumab therapy.[20] Others have continued to advocate immunohistochemical analysis over FISH for its lower cost and greater availability, proposing manual or digital-assisted subtraction of background HER2 immunostaining using benign epithelium as a way of improving HercepTest specificity.[18,21,22] Benign breast epithelium was available in only 54% of the HercepTest slides in one recent series, however.[23] Finally, proposals to subtract background epithelial staining have not clearly distinguished membrane-associated immunostaining, which may represent normal HER2 expression,[5,23,24] from cytoplasmic staining, widely regarded as nonspecific.[6,7,16]

One postulated advantage of immunohistochemical analysis over FISH for HER2 determination is the potential to detect protein overexpression in HER2-nonamplified tumors. Such overexpression-positive/amplification-negative tumors occur in 3% to 8% of breast cancers in most series,[25-27] but were as high as 29% and 31% in comparisons of the PathVysion (Vysis, Downers Grove, IL) FISH assay with the HercepTest immunohistochemical[23] assay and the Genentech clinical trials assays,[28] respectively. In a study of 900 breast cancers, however, Pauletti et al[14] found the immunohistochemical-FISH discrepant tumors behaved clinically as predicted by FISH. Finally, Tubbs et al[20] found that the immunohistochemically 2+/FISH-negative tumors contained no detectable HER2 messenger RNA (mRNA), indicating that up-regulation of HER2 gene transcription without gene amplification is unlikely. These studies indicate that the majority of immunohistochemically positive/FISH-negative tumors are false-positive immunohistochemical results.

Pauletti et al[25] and Kallioniemi et al[26] described the simultaneous enumeration of HER2 genes and chromosome 17 centromeres by FISH, defining HER2 gene amplification as the ratio of HER2 gene copies per chromosome 17 centromere. Two-color FISH is highly sensitive (96%-98%) and specific (100%) compared with Southern blot analysis. The FDA-approved FISH assay, PathVysion, also uses a dual-probe system for simultaneous enumeration of HER2 genes and chromosome 17 centromeres in FFPE breast tumors. Another FDA-approved commercial FISH assay, INFORM (Ventana Medical Systems, Tucson, AZ), defines HER2 gene amplification as a mean absolute HER2 gene copy number of more than 4 per tumor nucleus. A study by Pauletti et al[14] using the PathVysion FISH system in 900 breast cancer patients found chromosome 17 correction essential for demonstration of HER2 gene amplification; others believe it is unnecessary.[20]

We determined the HER2 status of a diverse group of primary breast cancer specimens using the HercepTest immunohistochemical and PathVysion FISH assays. The concordance between 2 experienced surgical pathologists (S.R.M., T.J.L.) in assessing immunohistochemical scores was determined. We also compared the HER2 gene and chromosome 17 (CEP17) copy numbers in relation to immunohistochemical score and attempted background subtraction using benign epithelium as has been proposed to improve the specificity of the immunohistochemical 2+ group.[18,21] Finally, we compared 2 definitions of HER2 gene amplification: absolute HER2 copies per tumor nucleus (threshold, 4.0) and HER2 copies per chromosome 17 centromere (threshold, 2.0).

Materials and Methods

Samples

The study used paraffinized tumor blocks from 215 breast cancers submitted for HER2 analysis from the hospitals and clinics of the Allina Health System (Minneapolis, MN), a diverse group of hospitals and outlying clinics in the upper Midwest. The specimens (11 from needle core biopsies, 72 from open or lumpectomy excisions, 49 from mastectomies, 31 from metastatic lesions, and 52 from unspecified

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Immunohistochemical Analysis

Immunohistochemical analysis was performed as specified by the manufacturer using the HercepTest kit. Briefly, 3-μm paraffin sections were placed in an oven overnight at 37°C. The slides were dewaxed in xylene, rehydrated in graded alcohol, incubated in citrate buffer at 95°C (in a water bath) for 20 minutes, and then washed in water for 5 minutes. The slides then were placed on an immunostainer (DAKO) using the primary polyclonal antibody and polymer detection system supplied by DAKO. Following immunohistochemical analysis staining, the slides were placed in hematoxylin for 1 minute, dehydrated in graded alcohol, cleared in xylene, and coverslipped.

Two pathologists independently scored slides as 0, 1+, 2+, or 3+ according to DAKO guidelines. Cytoplasmic staining was ignored. Only invasive tumor was scored. Scores of 0 or 1+ were regarded as immunohistochemically negative and 2+ or 3+ as immunohistochemically positive. Discrepant immunohistochemical scores were resolved at the 2-headed microscope. The consensus immunohistochemical score was used for comparison with the FISH results.

Fluorescence In Situ Hybridization

FISH was performed as specified by the manufacturer, with minor modifications. The PathVysion kit includes a SpectrumOrange-labeled DNA probe specific for the HER2 gene locus and a SpectrumGreen-labeled probe specific for the alpha satellite DNA sequence at the centromeric region of chromosome 17 (CEP17). Briefly, 4-μm-thick paraffin sections were cut on organosilane-coated slides, deparaffinized, pretreated in 0.2N hydrochloric acid and sodium thiocyanate as specified, then digested in protease solution at 37°C for 12 minutes (rather than 10, as specified in the package insert). After washing, the slides were fixed in neutral buffered formalin for 10 minutes, washed, dried, denatured in 70% formamide–standard saline citrate, and hybridized with the HER2/CEP17 dual-probe mixture. After posthybridization washing, the slides were dried, counterstained with 4,6-diaminido-2-phenylindole dihydrochloride (DAPI), and coverslipped. Finished slides were stored at -20°C and evaluated within 24 hours.

FISH slides were scored by 1 observer (J.S.) according to the manufacturer's guidelines. SpectrumOrange and SpectrumGreen signals were enumerated in 60 tumor nuclei. Total orange (HER2) and green (CEP17) signals were recorded, as was the HER2/CEP17 ratio. Small samples (such as core biopsies) containing fewer than 60 tumor nuclei were included if high quality, nonoverlapping tumor and normal nuclei were evident. Chromosome 17–corrected HER2 gene amplification was defined as an HER2/CEP17 ratio of 2.0 or more (as specified by Vysis). HER2/CEP17 ratios between 2.0 and 5.0, inclusive, were designated "low-copy" HER2-amplifed, while those greater than 5.0 were designated "high-copy" amplified. HER2 gene amplification using the absolute HER2 gene copy number was defined as a mean of 4.0 or more HER2 signals per tumor nucleus.[31] Modal CEP17 counts per 60 tumor nuclei were taken as the chromosome 17 copy number for each case, with modal CEP17 counts of 1, 2, and 3 or greater indicating chromosome 17 monosomy, disomy, and polysomy, respectively.

To examine the relationship between the immunohistochemical score and the chromosome 17 copy number of HER2-nonamplified tumors, we used both the Vysis-specified HER2/CEP17 ratio of 2.0 and a ratio of 1.8 as thresholds for amplification. The logic for including the lower HER2/CEP17 cutoff of 1.8 was that whereas the 2.0 cutoff is a clinical threshold determined by response to CAF (cyclophosphamide, doxorubicin, 5-fluorouracil) chemotherapy,[29] in theory, the HER2/CEP17 ratio of an HER2-nonamplified tumor should be near 1.0 (as the HER2 gene resides on chromosome 17). Thus, many tumors in the HER2/CEP17 ratio interval 1.8 to 2.0 may well be low-level HER2 gene–amplified.

Statistical Methods

FISH results were compared using total HER2 and/or CEP17 signals per 60 nuclei (or normalized to 60 when fewer tumor nuclei were available) and the *t* test method for comparison of means.

Results

Of 215 tumors, 10 were excluded for analytic failure (7 FISH, 3 immunohistochemical analysis) and 7 for insufficient tumor, leaving 198 samples for dual immunohistochemical and FISH analysis.

Pathologist concordance was 78% overall and 95%, 62%, 75%, and 83% for the 3+, 2+, 1+, and 0 immunohistochemical groups, respectively. When samples were grouped as immunohistochemical positive (2+, 3+) or immunohistochemical negative (0, 1+), pathologist concordance was 92% and 96%, respectively.

As classified by manufacturer guidelines, 86 (43.4%) of 198 cases were positive for HER2 overexpression by HercepTest (immunohistochemically positive) and 67 (33.8%) of 198 were positive for HER2 gene amplification by PathVysion (FISH-positive) ▯**Table 1**▯ and ▯**Image 1**▯. Thirty-four FISH-positive cases (51%) were low-copy HER2 gene–amplified, and 33 (49%) were high-copy amplified. All high-copy amplified cases were immunohistochemically

Table 1
Immunohistochemical Score and HER2/CEP17 Fluorescence In Situ Hybridization Ratio for 198 Breast Tumors*

	HER2/CEP17 Ratio			
Immunohistochemical Score	<2.0 (Non-amplified)	2.0-5.0 (Low-Copy Amplified)	>5.0 (High-Copy Amplified)	Total
0	51	2	0	53
1+	54	5	0	59
2+	26	14	5	45
3+	0	13	28	41
Total	131	34	33	198

CEP17, chromosome 17.
* The immunohistochemical score is the consensus score by 2 pathologists according to HercepTest guidelines. The ratio is for HER2/CEP17 signals in 60 tumor nuclei according to PathVysion guidelines. For proprietary information, see the text.

positive, while low-copy amplified cases occurred among all immunohistochemical groups (Table 1). All 41 3+ immunohistochemical cases were positive by FISH. Of 131 FISH-negative cases, 26 (19.8%) were immunohistochemically positive (all 2+). Of 67 FISH-positive cases, 7 (10.4%) were immunohistochemically negative (immunohistochemical score 0, 2 cases; immunohistochemical score 1+, 5 cases). Among the FISH-positive–immunohistochemically negative tumors, the HER2/CEP17 ratio was borderline (2.04-2.14) in 5 cases and 2.32 and 4.05 in 1 case each.

Consensus immunohistochemical scores correlated with mean HER2 gene copy number, mean CEP17 number, and the HER2/CEP17 ratio **Table 2**. Among 131 FISH-negative tumors, consensus immunohistochemical scores were weakly related to mean CEP17 copy number in a trend that approached statistical significance when the FISH threshold for amplification was lowered from 2.0 to 1.8 **Table 3**. Lowering the HER2/CEP17 cutoff to 1.8 removed 11 tumors from the nonamplified group as defined by the Vysis-specified clinical cutoff of 2.0.[29]

HER2 gene amplification as defined by absolute (mean HER2 signals per nucleus of 4.0 or more) and by chromosome 17–corrected (HER2/CEP17, 2.0 or more) criteria agreed in 178 (89.9%) of 198 breast tumors **Table 4**. Seventeen tumors classified as amplified by absolute criteria were nonamplified when chromosome 17–corrected. Three tumors were HER2-nonamplified by absolute criteria but were borderline amplified (HER2/CEP17 ratios of 2.02, 2.11, and 2.17) when corrected for chromosome 17.

Of 198 immunohistochemical slides, 105 (53.0%) contained sufficient benign epithelium to evaluate background immunostaining as suggested by Jacobs et al[18] and Lehr et al.[21] Of these 105 slides, 37 (35.2%) demonstrated weak to moderate epithelial-associated immunostaining that was unrelated to the respective tumor immunohistochemical score or FISH grouping (not shown). Of 26 immunohistochemically 2+/FISH-negative cases, 11 contained sufficient benign epithelium for background assessment, 4 of which demonstrated weak to moderate immunostaining. The pattern of HER2 immunostaining associated with normal epithelium was one that highlighted the basal and lateral cell membranes, while the luminal epithelial surfaces and surrounding myoepithelial cells were invariably immunonegative **Image 2**. Review of all 198 tumors revealed 20 with a

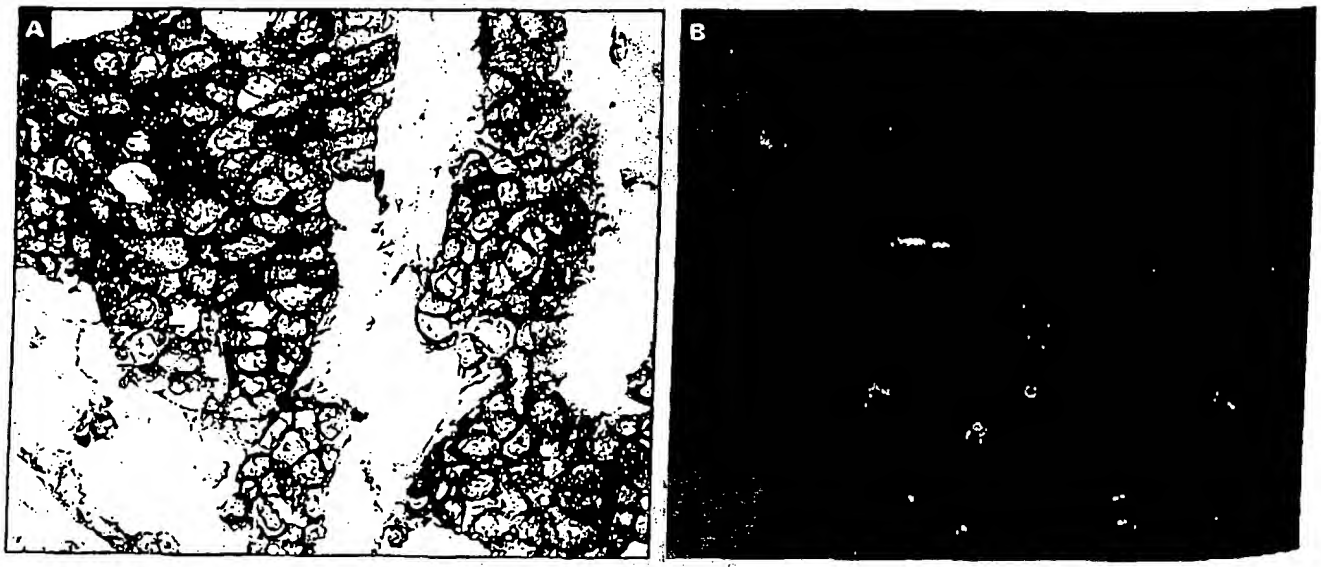

Image 1 **A**, Breast carcinoma, immunohistochemical score 3+, with dense, membrane-associated reaction indicating HER2 protein overexpression (HercepTest with hematoxylin counterstain, ×400). **B**, Corresponding 2-color fluorescence in situ hybridization image showing dense clusters of SpectrumOrange (red) signal indicating HER2 gene amplification. HER2/CEP17 ratio = 11.5 (×1,000). For proprietary information, see the text.

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similar basolateral pattern of immunostaining (Image 3); all were low to moderate grade, tubule-forming tumors with consensus immunohistochemical scores of 1+, 2+, 0, and 3+ in 12, 5, 2, and 1 tumor, respectively. Seventeen of 20 tumors with basolateral immunostaining were FISH-negative, while 3 were borderline FISH-positive (1 HER2/CEP17 ratio of 2.00 and 2 with ratios of 2.08).

Discussion

By using solid-matrix blotting methods and frozen section immunohistochemical analysis, Slamon et al[6,7] linked HER2 protein overexpression and gene amplification with aggressive clinical behavior in a subset of primary breast and ovarian cancers. Currently, HER2 assessment for clinical purposes uses immunohistochemical analysis and FISH, techniques that permit retrospective and cancer cell–specific analysis.[16] Following its FDA approval in 1998, reports of HercepTest false-positive rates approaching 50%[15] led to suggestions that FISH replace immunohistochemical analysis for clinical HER2 determination[14,25] or that HercepTest 2+ breast tumors be confirmed by FISH.[19] Tubbs et al[20] called for the FDA-mandated retraction of the HercepTest 2+ score as a criterion for trastuzumab therapy. Citing cost-effectiveness and the general availability of immunohistochemical analysis, Jacobs et al[18] and Lehr et al[21] proposed using benign epithelium to correct for background immunostaining as a way of improving HercepTest specificity. Background correction was impractical in our series, however, as only 105 (53.0%) of the tumor immunohistochemical slides included sufficient benign epithelium. Hoang et al[23] similarly reported that only 54% of their tumor HercepTest slides contained benign epithelium, although in their series, the normal epithelium appeared unstained. In our series, 37 of 105 immunohistochemical slides with benign epithelium showed detectable immunostaining in a delicate basal and lateral membrane pattern, with negative luminal surfaces. The basolateral pattern of immunostaining is consistent with reports of HER2 expression in benign epithelium as detected by light and electron microscopy.[24,30] It is unclear from the reports of Jacobs et al[18] and Lehr et al[21] whether the reported background epithelial HER2 immunostaining pertains to cytoplasmic reactivity, which is generally regarded as nonspecific, or membrane-associated staining, which may represent normal HER2 protein expression.[24]

The low level of HER2 protein associated with normal epithelium observed in our series may not have been detectable in immunohistochemical assays predating the HercepTest, which uses a potent polyclonal rabbit antibody reagent, heat-induced epitope retrieval, and polymer detection. A molecular basis for the basolateral pattern of normal HER2 immunostaining described herein is provided in a report describing a specific erbB-2 binding protein, ERBIN, which serves to localize the HER2 molecule to the basolateral membrane of mammalian epithelial cells.[5]

Table 2
HER2 and CEP17 Copies by Fluorescence In Situ Hybridization vs Immunohistochemical Score in 198 Breast Tumors*

Immunohistochemical Score	Mean HER2 per Nucleus	Mean CEP17 per Nucleus	HER2/CEP17 Ratio
0	2.67	2.38	1.17
1+	2.84	2.22	1.59
2+	6.98	2.84	2.27
3+	19.65	3.15	5.23

CEP17, chromosome 17.
* Mean HER2 and CEP17 signals per nucleus in 60 tumor nuclei. The immunohistochemical score is the consensus score by 2 pathologists according to HercepTest guidelines. The ratio is for HER2/CEP17 signals in 60 tumor nuclei according to PathVysion guidelines. For proprietary information, see the text.

Table 3
Immunohistochemical Score and CEP17 Copy Number by Fluorescence In Situ Hybridization in HER2-Nonamplified Breast Tumors*

Immunohistochemical Score	Mean CEP17 per Tumor Nucleus	Comparison Grouping	P†
HER2/CEP17 cutoff = 2.0 (n = 131)			
0	2.45 (n = 51)	0 vs 1	.82
1+	2.56 (n = 54)	1 vs 2	.42
2+	3.10 (n = 26)	0 vs 2	.15
		0 + 1 vs 2	.14
HER2/CEP17 cutoff = 1.8 (n = 120)			
0	2.45 (n = 49)	0 vs 1	.82
1+	2.56 (n = 51)	1 vs 2	.23
2+	3.46 (n = 20)	0 vs 2	.05
		0 + 1 vs 2	.04

CEP17, chromosome 17.
* The immunohistochemical score is the consensus by 2 pathologists according to HercepTest guidelines. For fluorescence in situ hybridization, the mean CEP17 signal copies in 60 tumor nuclei is given. The HER2/CEP17 ratio cutoff of 2.0 is according to PathVysion guidelines (threshold, ≥2.0). The modified HER2/CEP17 ratio cutoff has a threshold of ≥1.8. The immunohistochemical group with a score of 3+ was not compared because all 3+ tumors were HER2-amplified. For proprietary information, see the text.
† t Test.

Table 4
HER2 Gene Amplification Defined by HER2/CEP17 Ratio and Mean Absolute HER2 Gene Copy Number in 198 Breast Tumors*

Absolute HER2 Signals, Mean	HER2/CEP17 Ratio		
	<2.0 (–)	≥2.0 (+)	Total
HER2 ≥4.0 (+)	17 (+/–)	63 (+/+)	80
HER2 <4.0 (–)	115 (–/–)	3 (–/+)	118
Total	132	66	198

CEP17, chromosome 17; +, positive; –, negative.
* The ratio of HER2/CEP17 signals in 60 tumor nuclei. The mean absolute is the number of HER2 signals per tumor nucleus. Parentheses show result by absolute HER2 criteria followed by result by HER2/CEP17 ratio.

Review of our immunohistochemical slides disclosed 20 low to moderate grade, tubule-forming tumors with the basolateral pattern of immunostaining. These 20 tumors were predominantly immunohistochemically 1+ (12 cases) or 2+ (5 cases) and HER2-nonamplified (17 cases) or borderline amplified (2 HER2/CEP17 ratios of 2.08, and 1 of 2.00). It would be of interest to know whether the discontinuous, partial membrane staining associated with the classic immunohistochemical 1+ tumor staining pattern[15] reflects specific HER2 protein localization by the erbB-2 binding protein, ERBIN.

Two commercially available, FDA-approved FISH assays are available for the determination of HER2 gene amplification in FFPE breast tumors. These FISH assays differ in whether the threshold for HER2 gene amplification is defined by absolute (as in INFORM) or chromosome 17–corrected (as in PathVysion) HER2 gene copies. Although we did not compare these 2 commercial FISH assays, by applying the absolute threshold of 4.0 or more HER2 signals per nucleus to our series, we found 17 tumors with chromosome 17 polysomy that were HER2-amplified by absolute criteria, but HER2-nonamplified when chromosome 17–corrected. Eight of these 17 chromosome 17 polysomic tumors were immunohistochemically 2+ and were regarded as immunohistochemical false-positive results. It is unclear whether the HER2 protein observed in these tumors results from the additional chromosome 17 copies (and consequent extra HER2 genes), although among all HER2-nonamplified (FISH-negative) tumors, a weak association was found between immunohistochemical score and mean CEP17 copy number that was strengthened if the HER2/CEP17 ratio threshold for amplification was lowered from 2.0 to 1.8. Finally, 3 tumors (2 with immunohistochemical scores of 2+ and 1 with a score of 1+) that were HER2-amplified by chromosome 17 correction (HER2/CEP17 ratio, 2.0 or more) contained fewer than 4.0 mean HER2 genes per tumor nucleus; all had borderline HER2/CEP17 ratios (2.02, 2.11, and 2.17). These data corroborate the use of a chromosome 17 centromeric probe in correcting for chromosome 17 copy number changes when assaying for HER2 gene amplification by FISH.

The relationship between HER2 gene copy number, surface HER2 protein density, and clinical behavior is of biologic and clinical interest.[13-15] Any potential benefit from trastuzumab therapy among immunohistochemically 2+ breast tumors would likely be obscured in a clinical study as a result of the heterogeneity of HER2 gene status among the immunohistochemically 2+ group. Nevertheless, our data suggest that clinical trials using only the immunohistochemically 3+ standard to define HER2 overexpression will largely select for high-copy (>5 HER2 genes per nucleus) amplified tumors. Although exclusion of immunohistochemically 2+ tumors would eliminate many immunohistochemical false-positive results, in our series, 14 low-copy and 5 high-copy HER2-amplified tumors also would have been eliminated. The low-copy HER2-amplified tumors constitute a group that may have prognostic relevance[14] and can be accurately defined only by 2-color FISH using simultaneous probes for HER2 and chromosome 17. FISH assays simultaneously enumerating HER2 and chromosome 17 copies offer the greatest resolution in detecting breast tumors with alterations of the HER2 gene. As immunohistochemical methods cannot control for low-level immunostaining that may be normal or possibly the result of chromosome 17 polysomy, and as pathologist concordance for the 2+ group is poor (62% herein), the immunohistochemically 2+ group

Image 2 Benign epithelium in HercepTest immunohistochemical slide (tumor not shown). Basal and lateral cell membranes are stained; while luminal surfaces (with apical snouts) and surrounding myoepithelial cells are unstained (HercepTest with hematoxylin counterstain, ×1,000). For proprietary information, see the text.

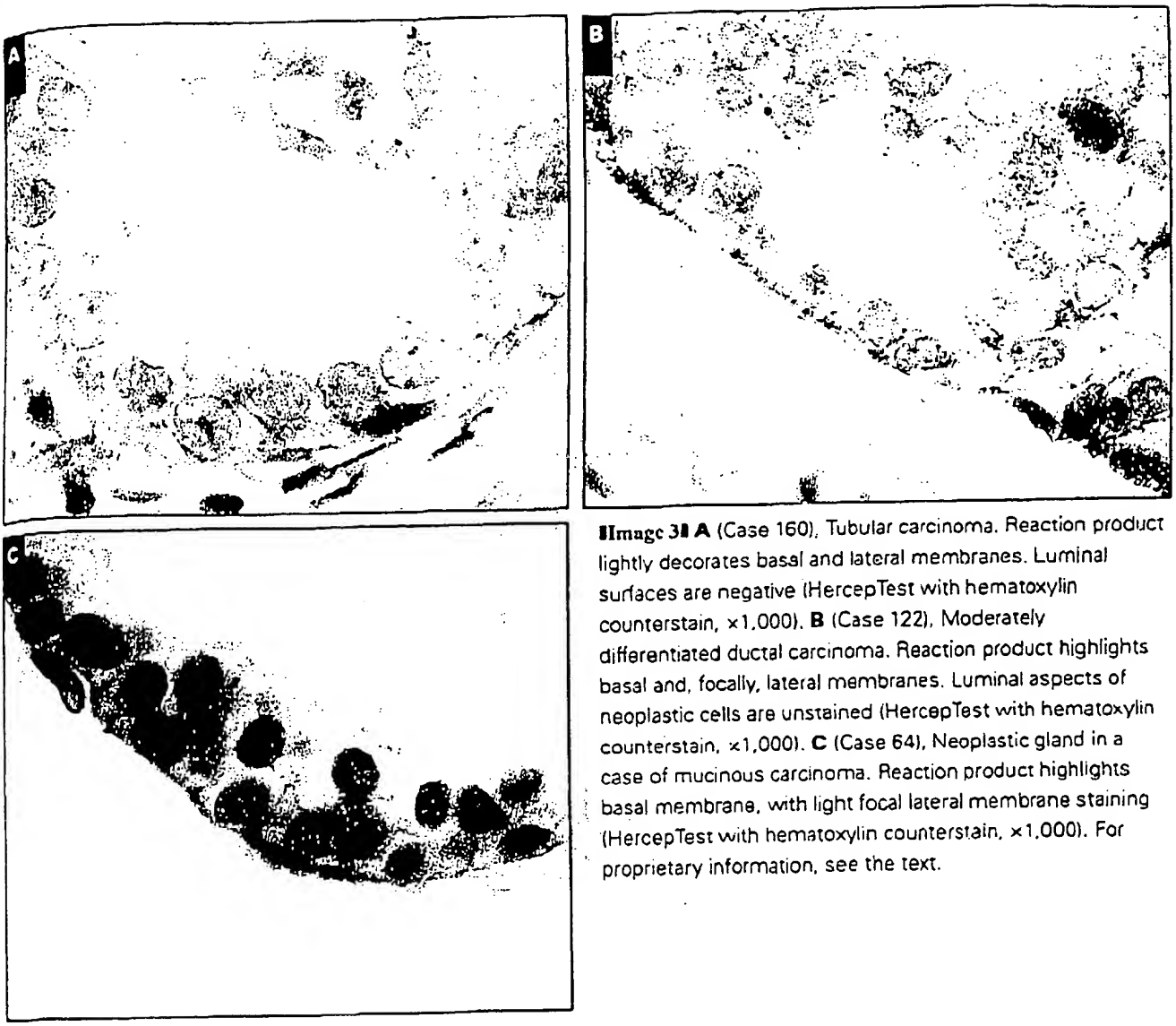

Image 3 **A** (Case 160), Tubular carcinoma. Reaction product lightly decorates basal and lateral membranes. Luminal surfaces are negative (HercepTest with hematoxylin counterstain, ×1,000). **B** (Case 122), Moderately differentiated ductal carcinoma. Reaction product highlights basal and, focally, lateral membranes. Luminal aspects of neoplastic cells are unstained (HercepTest with hematoxylin counterstain, ×1,000). **C** (Case 64), Neoplastic gland in a case of mucinous carcinoma. Reaction product highlights basal membrane, with light focal lateral membrane staining (HercepTest with hematoxylin counterstain, ×1,000). For proprietary information, see the text.

likely will remain biologically and clinically heterogeneous. The possibility that absolute HER2 copy number correlates better with HER2 protein expression level than chromosome 17–corrected copy number deserves further investigation.[31] A recent clinical study using simultaneous probes for the chromosome 17 centromere and HER2 gene found that the clinical outcome was related to absolute HER2 and to chromosome 17–corrected HER2 copy number.[14]

As in previous studies,[19,23] a strong concordance between FISH (using chromosome 17 correction) and immunohistochemical analysis was found for the most intensely positive (immunohistochemical score 3+) and negative (immunohistochemical score 0 or 1+) tumors but not for the immunohistochemically 2+ group (approximately 60% FISH-negative and 40% FISH-positive herein).

Of 33 tumors with discrepant immunohistochemical and FISH results, 26 were immunohistochemically positive and FISH-negative (all immunohistochemically 2+), and only 7 were immunohistochemically negative and FISH-positive (all low-copy HER2-amplified). The clinical significance of the immunohistochemically positive–FISH-negative tumors has been addressed recently in a large cohort (n = 900) of breast cancers, in which no outcome difference was found between the immunohistochemically positive–FISH-negative and the immunohistochemically negative–FISH-negative tumors.[14] In addition, a recent study of mRNA expression in breast tumors found the immunohistochemically 2+–FISH-negative tumors expressed no HER2 mRNA, suggesting the immunostaining of these single HER2 gene copy tumors was not the result of up-regulated HER2 gene transcription.[20]

The clinical significance of the rare (7 of 198) immunohistochemically negative–FISH-positive breast tumors is uncertain. In our series, all immunohistochemically negative–FISH-positive tumors were low-copy (2-5 HER2 signals per CEP17 signal) HER2-amplifed. Clinical studies using 2-color FISH to stratify tumors by absolute and chromosome

17–corrected HER2 copy number, with correlation to prognostic and therapeutic endpoints, should clarify the immunohistochemically negative–FISH-positive cases.

FISH and the HercepTest assay are highly concordant for immunohistochemically 3+ and negative (immunohistochemical score 0, 1+) breast tumors, although the immunohistochemically 2+ group includes both HER2-amplifed and HER2-nonamplified tumors. The mechanisms of HER2 expression among nonamplified tumors with immunohistochemical 1+ and 2+ immunostaining are unclear, but may reflect intact normal pathways of HER2 expression and membrane localization or may possibly involve chromosome 17 polysomy. Detection of normal levels of HER2 protein on benign epithelium and HER2-nonamplified tumors is not surprising given potency of the HercepTest assay. ERBIN, a receptor protein specifically localizing HER2 to the basolateral membrane,[5] may provide a molecular basis for understanding the microscopic patterns of immunostaining observed in association with benign epithelium and some tubule-forming, HER2-nonamplified breast tumors. Finally, when FISH is used to detect HER2 gene amplification, use of a chromosome 17 centromeric probe (as in the PathVysion kit) is crucial for distinguishing breast tumors with low-level HER2 gene amplification from those with chromosome 17 polysomy. It seems unlikely that adjustment of the immunohistochemical score using benign epithelium will improve the specificity of the HercepTest 2+ group. Continuing advances in early breast cancer detection and the use of smaller biopsy techniques are trends that will lead to smaller biopsy specimens, further reducing the amount of additional benign epithelium available as internal control tissue.

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